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14. ABSTRACT

TLR9 is a cellular DNA-receptor that is widely expressed in breast cancers. The aim of this work was to study whether DNA derived from dead breast cancer cells can induce invasion in living breast cancer cells. In addition to showing previously that "dead DNA" induces invasion *in vitro*, we discovered that TLR9 expression is hypoxia-regulated in breast cancer cells *in vitro* and *in vivo*. We also discovered that the lack of TLR9 results in a more aggressive tumor behavior in pre-clinical cancer models, but only in triple negative breast cancer cells. In line with these findings, we discovered that low TLR9 expression in breast tumors predicts poor prognosis, but only in patients with triple negative tumors. Our further pre-clinical experiments suggest that such poor prognosis may be due to an impaired immune-response at the tumor interface. Our results suggest that treatment induces the formation of DNA-structures which induces TLR9-mediated release of cytokines. Thus, our findings suggest that TLR9 expression and "dead DNA" are biologically important in triple negative breast cancer.

15. SUBJECT TERMS
None provided

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Table of Contents

INTRODUCTION

 Toll like receptor-9 (TLR9) is a cellular DNA-receptor which is widely expressed in breast cancers. We have shown previously that stimulation of TLR9 with endogenous TLR9-ligands induces their invasion. The contribution of TLR9 to breast cancer pathophysiology has been, however, unknown. Our hypothesis entering these studies was that DNA, which is derived from dying cancer cells can serve as an endogenous TLR9-ligand and induce TLR9-mediated invasion and thereby, contribute to the poor prognosis in some types of breast cancers.

BODY:

Aim #1. Study the mechanism how apoptotic DNA induces breast cancer invasion in vitro and in vivo.

1.1 Study the mechanism how apoptotic DNA stimulates breast cancer invasion in vitro.
1.2 The role of DNA receptor expression in breast cancer growth and metastasis in vivo.

The TLR9 siRNA cells have been established and described in the attached publication (Appendix 1). We have also established work with siRNA AIM2 breast cancer cells, but no specific experiments have been done with these cells yet. We have only begun to perform the experiments with the DAI-siRNA cells. In the above mentioned publication, we demonstrate that the TLR9 siRNA tumors in the triple negative background grow more aggressively than the control siRNA cells. This was a surprise finding and went against our expectations. No such difference in tumor growth rates was detected between ER+ breast cancer cells (control or TLR9 siRNA). Within the manuscript (Appendix 1) we established that TLR9 expression is highly hypoxia-regulated and that in the absence of TLR9 expression in hypoxia, some other protein mediates the increased invasion. Our further hypothesis is that TLR7 is the critical protein in this context. We also investigated treatment-responses *in vivo* and compared those in the triple negative control and TLR9 siRNA tumors. We discovered that although there was a trend for smaller % decrease in tumor size in response to doxorubicine-treatment in the TLR9 siRNA-tumors, there was no statistically significant differences in the tumor responses (Fig. 1 a – b). However, interestingly, the mice that had control siRNA tumors lost twice as much weight than the mice that were inoculated with the TLR9 siRNA tumors (Fig. 1 c – d). Since the mice weights were similar in the vehicle-treatment groups, we hypothesized that doxorubicine-treatment induces the formation of DNA-structures which are released from dying cancer cells. When these DNA-structures are taken up via living cancer cells, a TLR9-mediated inflammation takes place, resulting in the release of TNF-α and other cytokines which might explain the weigh loss. We confirmed that the weight loss is due to a difference in lean mass, thus suggesting that it is cachexia (Fig. 1 e). Furthermore, we hypothesized that DNA from doxorubicine-killed breast cancer cells could induce cytokine release from living breast cancer cells. We discovered that this is the case indeed (Fig. 2). We have yet to show that this effect is TLR9-mediated.
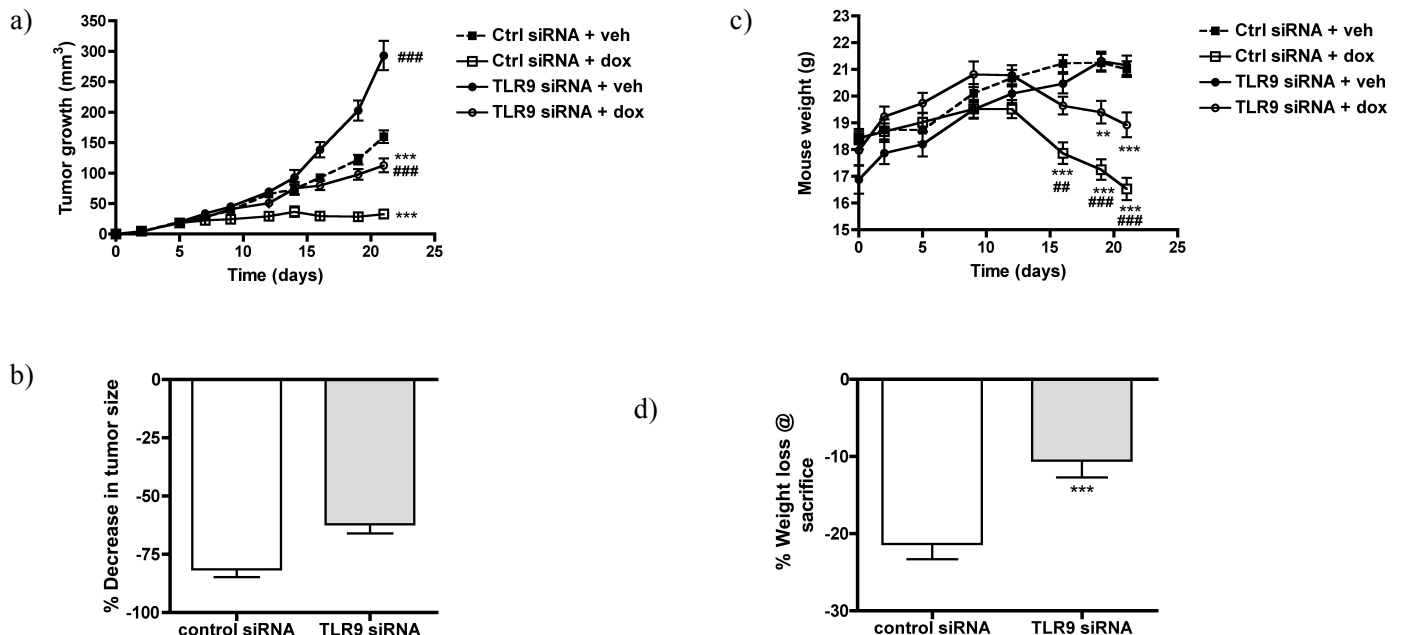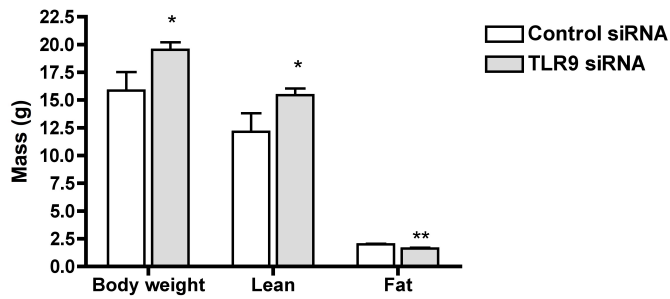
e)

Figure 1. Nude mice were inoculated with control or TLR9 siRNA MDA-MB-231 cells and the mice were treated with vehicle or doxorubicine. a) TLR9-siRNA cells formed larger tumors (### $p<0.001$ vs. ctrl siRNA tumors, *** $p<0.001$ vs. vehicle-treatment) but the response to treatment b) was similar in both groups. Data is shown as % decrease in tumor size, as compared with vehicle-treated tumors at sacrifice, n = 30, mean ± sem.
c) Mouse weights during the course of the experiment. Although vehicle-treated mice had similar weights, doxorubicine-treatment induced a more profound weight loss in mice that were inoculated with the control siRNA cells (n=30, mean ± sem, ### $p<0.001$ vs. TLR9 siRNA, ** $p<0.01$, *** $p<0.001$ vs. vehicle-treatment). d) % Weight loss at sacrifice, as compared with weights of vehicle-treated mice in corresponding groups at sacrifice. Mean ± sem, n=30, *** $p<0.001$ vs. control siRNA group. e) Weights of doxorubicine-treated mice, as determined by DEXA. The increased body weights of the TLR9 siRNA-tumor bearing mice are due to increased lean mass (n=5, mean ± SD, * $p<0.01$ vs. mice bearing control siRNA tumors).
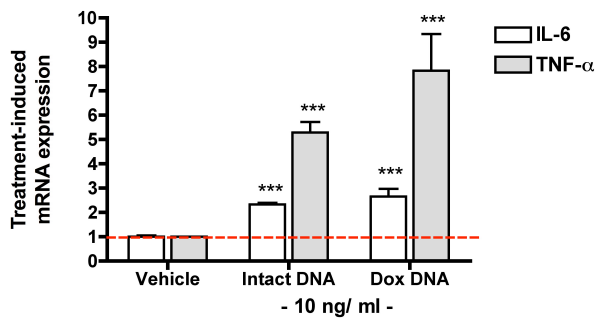

Figure 2. MDA-MB-231 breast cancer cells were cultured in the presence of 10 ng/ml of DNA from intact (isolated from proliferating breast cancer cells) or doxorubicine-killed (Dox DNA) breast cancer cells. IL-6 and TNF−α mRNA were measured 24 h later from the treated cells with qRT-PCR. Mean ± sd, n=12, *** $p<0.001$ vs. vehicle treatment.

We also followed tumor regrowth in mice that had been treated with doxorubicine. It was noted that the TLR9 siRNA tumors in the triple negative background grew back rapidly whereas control siRNA tumors remained small (Figure 3).
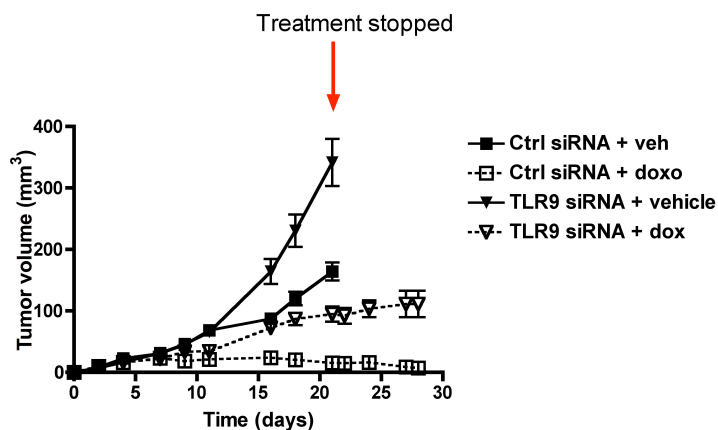

Figure 3. Tumor regrowth was followed in mice that were doxorubicine-treated. N= 10 (after vehicle-treated mice were sacrificed). Mean ± sem, n=10.

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We did similar *in vivo* studies also using the ER+ T47-D cells. In these cells, the tumor response to doxorubicine-treatment appears to be significantly less in the TLR9 siRNA tumors (Fig. 4). We do not, however, consider these results conclusive yet as they have only been done once, with a small number of mice. Also, the mouse weights in this study were not recorded so we will repeat the experiment.
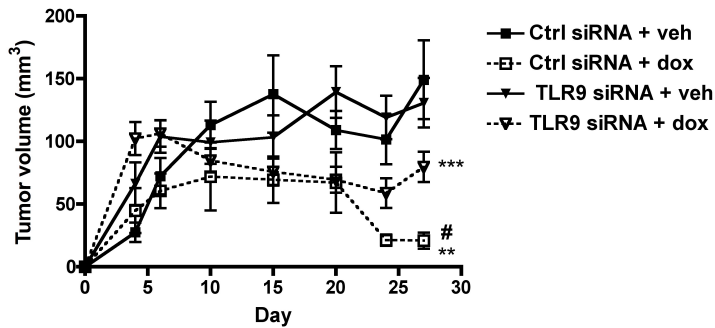
a)

Figure 4. Nude mice were inoculated with control siRNA or TLR9 siRNA cells in the ER+ T47-D background. The mice were treated with vehicle or doxorubicine and tumor volume was followed. a) There was no difference in the tumor sizes of vehicle-treated groups, but doxorubicine induced a more profound decrease in tumor growth of the control siRNA cells, mean ± sem, n=6-10, ** $p<0.01$, *** $p<0.001$ vs. vehicle, # $p<0.05$ vs. doxorubicine-treatment. B) % decrease in tumor size in response to doxorubicine-treatment, as compared against the mean tumor size of vehicle-treated tumor at sacrifice. Mean ± sem, n=6-10, ** $p<0.01$ vs. ctrl siRNA tumors.

b)
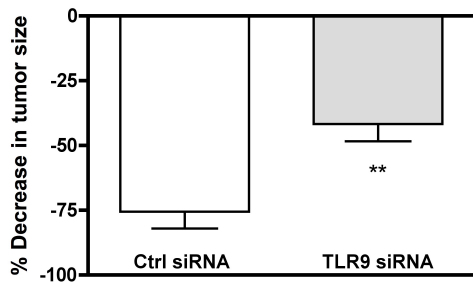
Conclusions from the experiments done so far in the Specific Aim # 1: Tumor TLR9 expression has a dramatic effect on prognosis in triple negative breast cancers. The lack of TLR9 in the triple negative tumors results in poor prognosis because 1) in the absence of TLR9, the cancer cells become highly invasive and viable in hypoxia and 2) the tumors lacking TLR9 possibly do not evoke a strong enough of an anti-tumor immune response that would result in the destruction of residual disease. We will continue to work on this topic to find a cure for these patients. In addition, our results suggest that "dead DNA"-induced and TLR9-mediated invasion takes place when large amounts of DNA are available for the viable cancer cells. Although not detected in the pre-clinical models used here, this mechanism may be important in other models of cancer.

Specific Aim # 2: Study the importance of apoptotic microparticles as a delivery mechanism for DNA.

This aim has been transferred to the extension period of the grant.

Specific Aim # 3: Reported separately.

KEY RESEARCH ACCOMPLISHMENTS: Bulleted list of key research accomplishments emanating from this research.

1) We have discovered a novel, poor prognosis subgroup of triple negative breast cancer as defined by tumor TLR9 expression.
2) Based on our results, we have formulated two further hypotheses as to why these patients do poorly.

REPORTABLE OUTCOMES:

Manuscripts: Tuomela J. et al. "Low TLR9 expression defines an aggressive subtype of triple negative breast cancer", 2012, Breast Cancer Research and Treatment, In Press, PMID: 22847512

Abstracts: Tuomela J. et al. Low Toll−Like Receptor 9 Expression Is Required for the Aggressive Behavior of Triple Negative Breast Cancer Cells in Hypoxia, PROGRAM #P2−11−10, San Antonio Breast Cancer Symposium, San Antonio, TX, December 2011

Degrees obtained that are supported by this award; Jouko Sandholm, PhD (pending)

Funding applied for based on work supported by this award: Susan Komen grant (pending), RO1 (pending), DOD (pending)

Grants received: from the UAB Comprehensive Cancer Center to study the prognostic role of TLR9 expression in African-American breast cancer patients.

Employment or research opportunities applied for and/or received based on experience/training supported by this award: Dr. Johanna Tuomela, PhD, post-doctoral fellow, Jouko Sandholm, M.Sc., graduate student.

CONCLUSION: We have determined that DNA, which is derived from chemotherapy-killed breast cancer cells has important biological effects on living breast cancer cells and these effects induced by the "Dead DNA" may determine the course of the disease in a dramatic way, especially in triple negative breast cancer. First, we demonstrate that such "dead DNA" is taken up into living breast cancer cells and that complexing with LL-37 increases this cellular up-take. Second, "dead DNA" induces *in vitro* invasion in various breast cancer cells, also in brain cancer cells and this invasion is TLR9-mediated and due to TLR9-regulated activation of proteolytic enzymes. This effect, however, requires a lot of DNA and it is unclear whether or not such an invasion takes place *in vivo*. It does not seem to do so in the animal models that we have used, but this phenomenon may be important in other cancers where more DNA is released upon cell death. Our results suggest that the pathophysiological significance of the dead DNA-induced invasion should be studied in an animal model that better mimics cancers that are undergoing rapid cell death and where large amounts of "dead DNA" are available in the vicinity of the tumor.

We also discovered that such "dead DNA" has pro-inflammatory effects in living cancer cells. Our animal experiments suggest that these effects are also TLR9-mediated, although we have to still characterize the findings further. These findings may partly explain why the low TLR9-triple negative subgroup of breast cancer patients, which we have first described (Tuomela et al. 2012) has such poor prognosis. We also demonstrated that TLR9 expression is hypoxia-regulated and in the absence of hypoxia-induced TLR9 expression, triple negative, but not ER+ breast cancer cells, become highly invasive.

We are now seeking further funding to test the hypotheses derived from the current work.

SO WHAT: We set out to test the hypothesis that DNA from dead cancer cells induces invasion in living cancer cells and that this "death-induced vicious cycle" contributes to tumor spread and metastasis in breast cancer. While we have shown that this indeed is the case *in vitro*, we have been unable to demonstrate that it is important for metastasis of small breast cancers *in vivo*. However, while doing these experiments, we ended up discovering a novel, poor-prognosis subgroup of breast cancer patients that have low tumor TLR9 expression + triple negative tumors. These patients may represent as many as 10 % of all breast cancer patients. Interestingly, however, the experiments proposed in the original grant may

have provided further clues as to why these patients have such poor prognosis. Based on the findings done with this grant money, we have a reason to believe that in addition to inducing invasion, "dead DNA" also induces TLR9-mediated inflammation and actually, it is this inflammatory reaction, which takes place at much smaller DNA concentrations than invasion, is critical for the prognosis.

Our results imply that tumor TLR9 expression and "dead DNA"-TLR9 interaction at the tumor interface are critical determinant of prognosis in a subgroup of breast cancer patients that may account for as many as 10 % of all breast cancers.

In our future work, we will test the hypotheses that are based on results from this grant.

PRECLINICAL STUDY

Low TLR9 expression defines an aggressive subtype of triple-negative breast cancer

Johanna Tuomela · Jouko Sandholm · Peeter Karihtala · Joanna Ilvesaro ·
Katri S. Vuopala · Joonas H. Kauppila · Saila Kauppila · Dongquan Chen ·
Christine Pressey · Pirkko Härkönen · Kevin W. Harris · David Graves ·
Päivi K. Auvinen · Ylermi Soini · Arja Jukkola-Vuorinen · Katri S. Selander

Abstract Toll-like receptor-9 (TLR9) is a DNA receptor widely expressed in cancers. Although synthetic TLR9 ligands induce cancer cell invasion in vitro, the role of TLR9 in cancer pathophysiology is unclear. We discovered that low tumor TLR9 expression is associated with significantly shortened disease-specific survival in patients with triple negative but not with ER+ breast cancers.

Johanna Tuomela, Jouko Sandholm, and Peeter Karihtala have equally contributed to this study.

A likely mechanism of this clinical finding involves differential responses to hypoxia. Our pre-clinical studies indicate that while TLR9 expression is hypoxia-regulated, low TLR9 expression has different effects on triple negative and ER+ breast cancer invasion in hypoxia. Hypoxia-induced invasion is augmented by TLR9 siRNA in triple negative, but not in ER+ breast cancer cells. This is possibly due to differential TLR9-regulated TIMP-3 expression, which remains detectable in ER+ cells but disappears from triple-negative TLR9 siRNA cells in hypoxia. Our results demonstrate a novel role for this innate immunity receptor in cancer biology and suggest that TLR9 expression may be a novel marker for triple-negative breast

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cancer patients who are at a high risk of relapse. Furthermore, these results suggest that interventions or events, which induce hypoxia or down-regulate TLR9 expression in triple-negative breast cancer cells may actually induce their spread.

Keywords Toll-like receptor-9 · Triple negative · Hypoxia · Invasion · Breast cancer

Introduction

Toll-like receptors (TLRs) are pattern recognition proteins that detect both microbe- and host-derived molecular patterns [1, 2]. At least 13 mammalian TLRs with specific ligands have been recognized so far. For example, TLR4 is the receptor for bacterial lipopolysaccharide (LPS). TLR5 recognizes flagellin, and members of the Toll-like receptor-9 (TLR9) subfamily are RNA (TLRs 3, 7, and 8) and DNA (TLR9) receptors [1–3]. In addition to the microbe-derived ligands, several endogenous ligands demonstrating receptor specificity have also been described for the various TLRs [4, 5]. The sub-cellular expression of TLRs varies. While TLRs 1, 2, and 4 are typically expressed on the cell surface, TLR3, TLR13, and the TLR9 subfamily members reside within intracellular compartments [3, 6–8]. Ligand binding to TLRs activates the transcription factor NF-κB; in cells of the immune system, the eventual outcome of TLR activation is an innate immune reaction characterized by increased production of inflammatory mediators [2].

Recent studies indicate that TLR9 is frequently expressed in various cancer cell lines in vitro, including breast, brain, gastric, lung, and prostate cancer cells [8–13]. We and others have demonstrated that stimulation of TLR9-expressing cancer cells with synthetic TLR9 ligands (CpG sequence-containing oligonucleotides) increases their in vitro invasion, with associated down-regulation of tissue inhibitor of matrix metalloproteinase-3 (TIMP-3) and up-regulation of matrix metalloproteinase-13 (MMP-13) activity [9, 10, 13, 14]. This effect is mediated via TLR9 and TRAF6, but not via MyD88 [10, 14]. TLR9 expression has also been demonstrated in various clinical tumor specimens, especially in breast and prostate cancers [9, 15–19]. In breast cancers, high TLR9 expression has been associated with estrogen receptor-negative (ER−) status and poor differentiation of the tumors [15, 17]. Nevertheless, both high and low TLR9 expression can be found in all of the clinically significant subgroups of breast cancer, including estrogen (ER) and progesterone (PgR) receptor-positive, human epidermal growth factor receptor-2 (HER2)-positive, and triple-negative tumors (which lack the expression of all these receptors) [15]. Despite the well-documented expression of TLR9 in various cancers and the invasive response to TLR9 ligands in vitro, the contribution of this protein to cancer pathophysiology is unclear.

Results

Triple-negative TLR9 siRNA breast cancer cells are less invasive in vitro but form larger tumors in vivo

In order to study the role of TLR9 in breast cancer pathophysiology, we used human, triple-negative MDA-MB-231 breast cancer cells as a model because these cells express high levels of TLR9 and treatment with synthetic TLR9 ligands increases their invasion [10, 14]. Also, because the natural, invasion-inducing ligand for TLR9 is currently unknown, we chose to study the role of the TLR9 protein per se in these experiments, in the absence of exogenous TLR9 ligands. TLR9 expression was first down-regulated in MDA-MB-231 cells through a stable, plasmid-based siRNA approach [14]. Characterization of these cells in normoxic, steady-state conditions is shown Fig. 1. In brief, as compared with the control siRNA cells, the TLR9 siRNA cells exhibited decreased TLR9 mRNA expression by ∼33 %, which was associated with decreased MMP-2, MMP-9, and MMP-13 mRNA and increased TIMP-3 mRNA expression (Fig. 1a). TLR9 and TIMP-3 protein expression levels demonstrated similar, slight changes (Fig. 1b). Relative to the control siRNA cells, the TLR9 siRNA cells also exhibited significantly increased expression of TIMP-1 and TIMP-2 mRNAs (Supplementary Fig. S1). Consistent with this anti-invasive gene expression profile, the TLR9 siRNA cells were indeed significantly less invasive than the control siRNA cells, suggesting that the overall ratio of proteolytic enzyme activity to their inhibitors favors an anti-invasive phenotype of the TLR9 siRNA cells in normoxic culture conditions (Fig. 1c). However, when the cells were inoculated into the mammary fat pads of nude mice, the TLR9 siRNA cells behaved more aggressively and formed significantly larger tumors than the control cells (Fig. 1d). Maintenance of the lower TLR9 mRNA expression level throughout the animal experiment was confirmed with qRT-PCR (Supplementary Fig. S2).

Breast cancer patients with triple-negative tumors and decreased tumor TLR9 expression have significantly shortened disease-specific survival

We then studied the correlation of TLR9 expression with breast cancer-specific survival in a clinical breast cancer cohort. The basal, clinicopathological parameters of the analyzed tumors are shown in Supplementary Table S1.
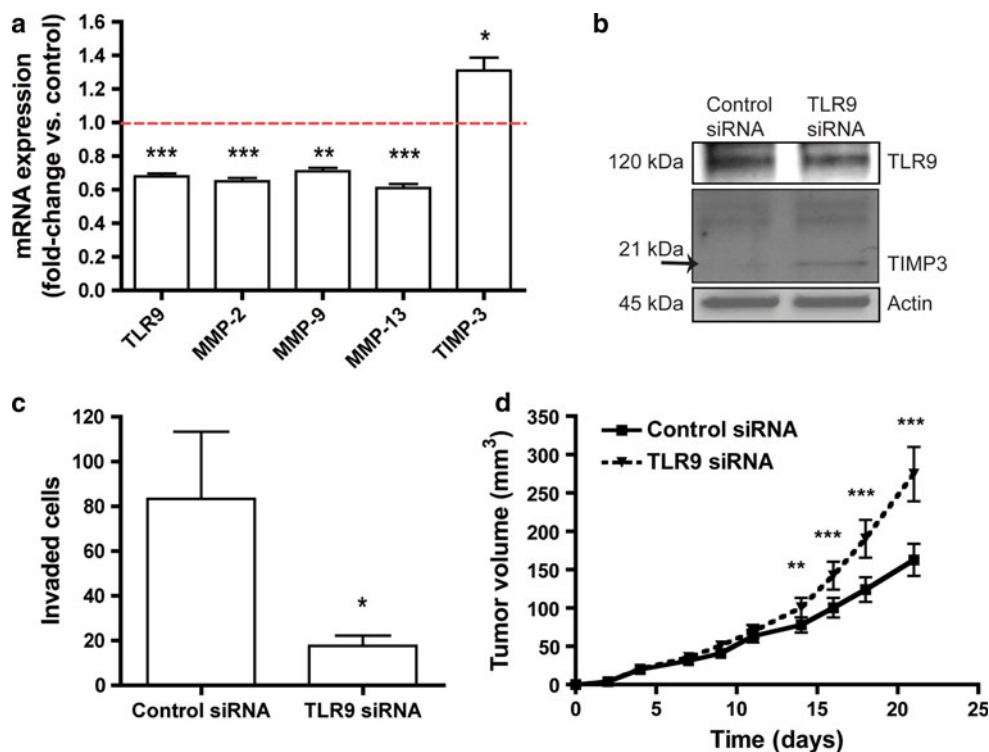
Fig. 1 a Characterization of the stable control siRNA and TLR9 siRNA MDA-MB-231 cells in normoxia. The *bars* represent changes in the indicated mRNA levels in the TLR9 siRNA cells, relative to control siRNA cells. The *dotted line* represents the expression levels of the mRNAs in the control cells. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ versus control siRNA cells. Mean $\pm$ SD, $n = 6$. **b** TLR9 and TIMP-3 protein expression levels in normoxia in the corresponding cells. The biggest change in TIMP-3 expression was seen at the 21 kDa band (*arrow*). The same blot was stripped and re-blotted with the anti-actin antibody, to show equal loading. **c** The basal invasion capacity of the control siRNA and TLR9 siRNA cells in normoxia, data is expressed as mean $\pm$ SEM, $n = 6$, $*p < 0.05$ versus control siRNA cells. **d** Orthotopic growth of the control and TLR9 siRNA tumors in nude mice in vivo, indicated by tumor growth curves as a function of time, $n = 60$ (tumors, data is combined from three separate in vivo experiments). $**p < 0.05$, $***p < 0.001$ TLR9 siRNA versus control siRNA tumors

TLR9 expression in these tumors was characterized with immunohistochemistry, as previously shown by us [15]. TLR9 expression was not associated with breast cancer-specific survival in the group of patients that had ER+/PgR+/HER2-tumors (Fig. 2a). However, among triple-negative (ER−, PR−, HER2−) breast cancer patients, low tumor TLR9 expression was associated with a significantly shortened disease-specific survival (Fig. 2b). Although the patients with both low and high TLR9-expressing triple-negative tumors were similar with regard to standard clinical prognostic factors, such as tumor size, grade, and nodal status, (Supplementary Table S1), 18 of 48 (37.5 %) patients with low tumor TLR9 expression died of breast cancer during the follow-up time, whereas only 4 of 51 (7.8 %) did so in the higher TLR9 score group. High tumor TLR9 expression was, however, significantly associated with the absence of lymphatic vessel invasion and lower proliferation rate. Taken together, these data suggest that low TLR9 expression may be a novel poor prognosis marker among patients with triple-negative breast cancers. The results further suggest that although the low TLR9 expressing triple-negative breast cancer cells are less invasive in normoxia in vitro, their behavior changes dramatically to very aggressive in vivo and this switch in their behavior may also explain the poor breast cancer-specific survival of triple-negative breast cancer patients with low tumor expression of TLR9.

Hypoxia induces TLR9 expression in breast cancer cells

While characterizing the reasons why low TLR9 expression would result in aggressive behavior of triple-negative breast cancer cells, we chose to investigate the role of hypoxia in this process. Hypoxia is a fundamental characteristic of solid tumors, which induces various adaptive changes in cellular functions, eventually leading to increased tumor growth, invasion, and metastasis [20]. Interestingly also, we noticed that the growth rates of the control and TLR9 siRNA tumors started to separate after day 12 when especially the TLR9 siRNA tumor diameters exceeded ~ 5 mm, being past the point at which tumors have been shown to become
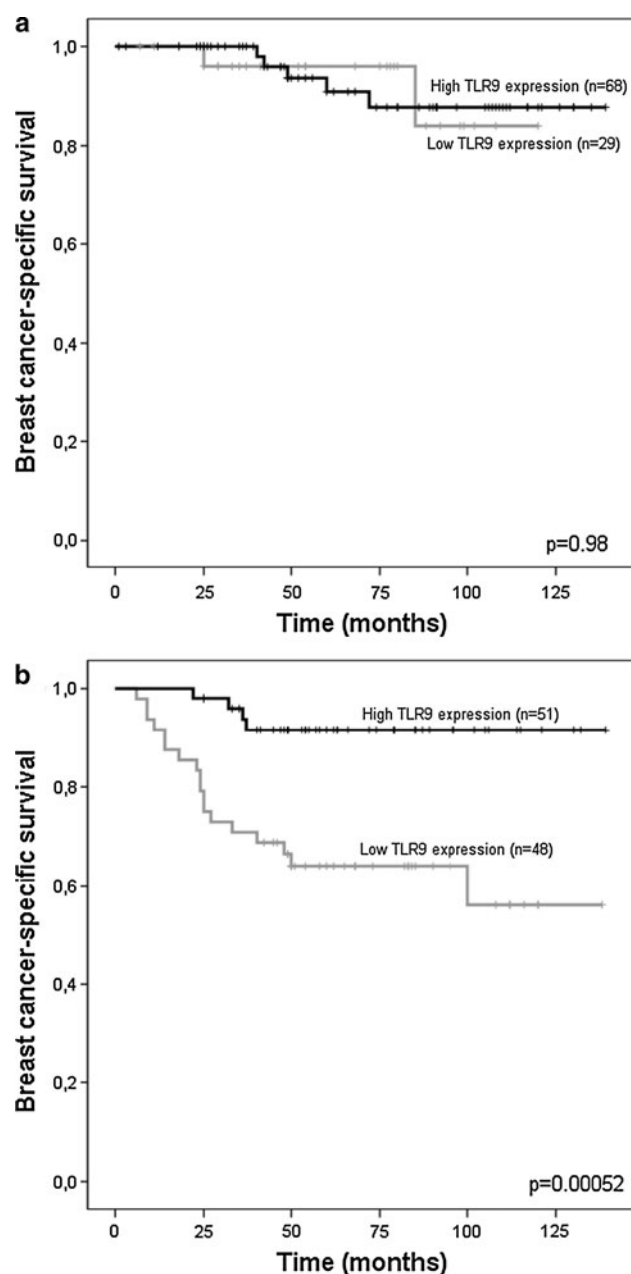
Fig. 2 Kaplan–Meier curves showing breast cancer-specific survival according to immunohistochemical TLR9 expression level in **a** ER+/PR+/HER2− breast cancers (log-rank $p = 0.98$) and **b** triple-negative (ER−/PR−/HER2−) breast cancers (log-rank $p = 0.00052$)

hypoxic [21]. Among breast cancers, especially those with ER and PR-negative status have been associated with hypoxia [22, 23]. Hypoxic tissue conditions also up-regulate the expression of other TLRs in normal tissues [24]. With these previous findings in mind, we hypothesized that hypoxia may affect TLR9 expression and invasion in breast cancer cells. Expression of TLR9 and MMP-13 were indeed markedly up-regulated within and immediately adjacent to the necrotic (and thus hypoxic) core of the subcutaneous tumors formed by MDA-MB-231 cells (Fig. 3a). Culture of

MDA-MB-231 cells in hypoxia in vitro also increased their TLR9 and MMP-13 mRNA expression after 24 h, while expression of TIMP-3 mRNA, an important regulator of MMP activity was significantly down-regulated at the corresponding time points and almost completely gone after 24 h (Fig. 3b). The protein levels of TLR9 and TIMP-3 paralleled the mRNA levels (Fig. 3c). In addition, MMP-2 and MMP-9 mRNAs were also significantly up-regulated by hypoxia at 24 h. Furthermore, there were also slight, but statistically significant changes in the mRNA expression levels of other TIMPs: TIMP-1 mRNA was slightly up-regulated and TIMP-2 mRNA expression was slightly down-regulated by hypoxia (Fig. 3d). Finally, increased proteolytic activity was detected with zymograms in the supernatants of MDA-MB-231 cells that had been cultured in hypoxia, as compared with normoxic supernatants. Taken together, these results suggest that the overall expression ratio of MMPs to their inhibitors favors proteolysis in hypoxia in these cells (Fig. 3e).

Hypoxia-induced up-regulation of TLR9 is mediated via HIF-1α

Hypoxia-inducible factor-1α (HIF-1α) is the primary regulator of response to acute hypoxia [25]. HIF-1 was recently shown to induce TLR2 and TLR6 expression in various benign cells under hypoxic conditions [24]. Therefore, we determined the role of HIF-1α in the hypoxia-induced up-regulation of TLR9 expression in MDA-MB-231 cells. The cells transfected with HIF-1α, TLR9, or non-targeting control siRNA oligonucleotides were cultured for 24 h under hypoxia or normoxia. In the control siRNA cells, hypoxia induced a significant increase in both HIF-1α and TLR9 mRNA expression (Fig. 4a). In the HIF-1α siRNA cells, the expression of both hypoxia-induced HIF-1α and TLR9 mRNAs were blunted. In cells transfected with TLR9 siRNA oligonucleotides, hypoxia-induced TLR9 mRNA expression was blunted, but HIF-1α mRNA was not. The results were also similar at the protein level (Fig. 4b, c). Taken together, these results suggest that TLR9 is a downstream target for HIF-1α in the MDA-MB-231 cells in hypoxia.

Hypoxia-induced invasion is significantly enhanced in triple negative, but not in ER+ TLR9 siRNA breast cancer cells

Cancer cells are known to become more invasive in hypoxia [26]. Considering the previous results about the role of TLR9 in cancer cell invasion in vitro, we next investigated whether TLR9 expression per se also has a role in hypoxia-induced invasion [9, 10, 14, 26]. As expected, parental MDA-MB-231 cells became more
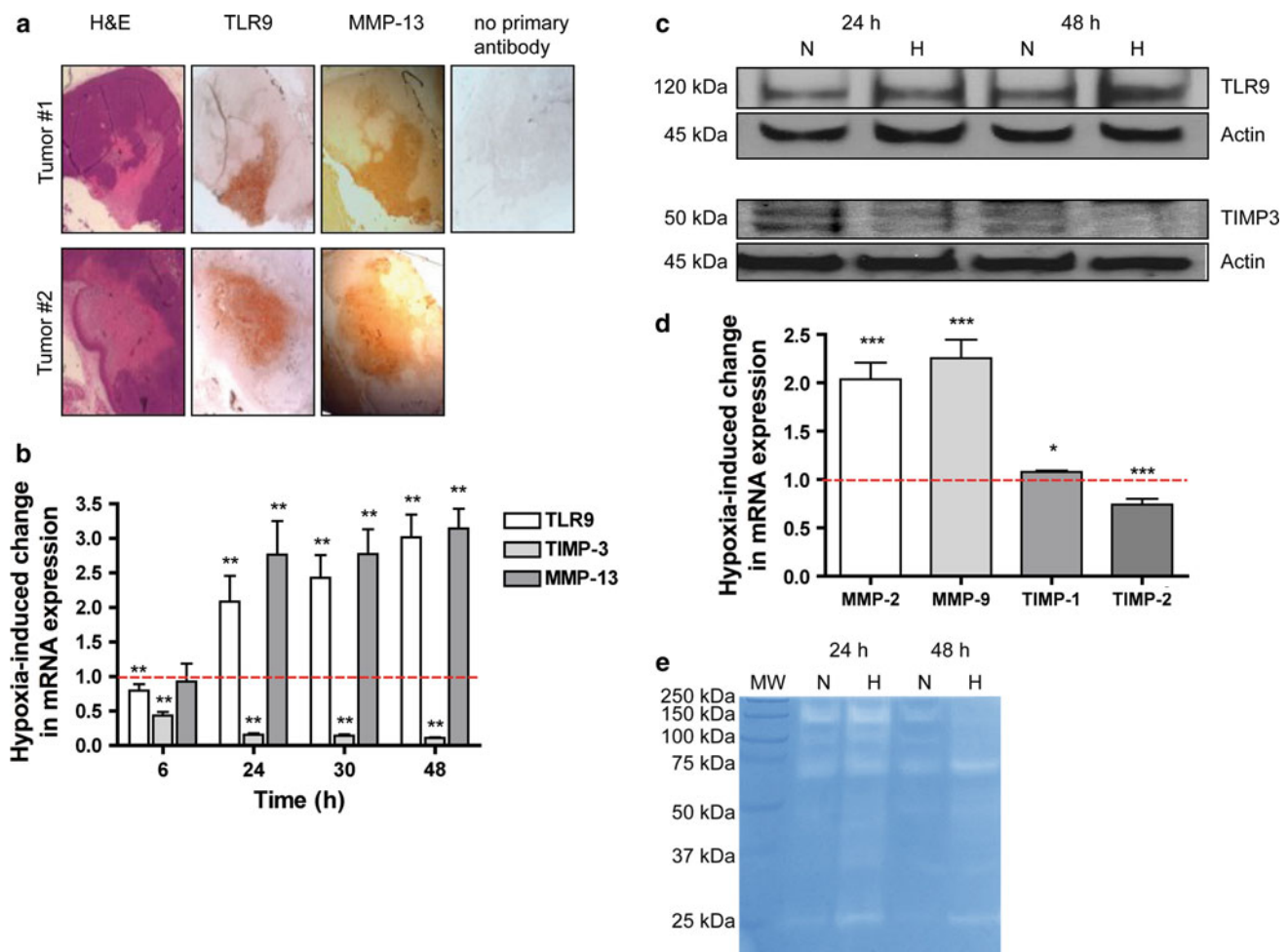
Fig. 3 a MDA-MB-231 cell tumors (1 and 2) that were grown orthotopically in nude mice were processed for histology and adjacent sections were subjected to H&E, and anti-TLR9 and anti-MMP-13 immunohistochemical stainings. The *pink areas* in the H&E-stained sections represent necrosis, the *purple areas* represent viable tissue. TLR9- and MMP-13 stainings overlap with the necrotic area. Primary antibody was omitted from the negative staining control. **b** MDA-MB-231 cells were cultured for the indicated times in hypoxia and normoxia in vitro and TLR9, TIMP-3, and MMP-13 mRNA expression were measured with qRT-PCR. The *bars* represent hypoxia-induced changes, relative to normoxia (*dotted line*), mean ± SD, $n = 6$ **$p < 0.01$ versus the corresponding value in normoxia. **c** Western blot of TLR9 and TIMP-3 from MDA-MB-231 cell cultured in normoxia (N) or hypoxia (H) for the indicated times. Actin bands of the same, stripped blots are shown to indicate equal loading. **d** Hypoxia-induced changes of MMP-2, MMP-9, TIMP-1, and TIMP-2 mRNAs from MDA-MB-231 cells at 24 h. The bars represent hypoxia-induced changes, relative to normoxia, mean ± SD, $n = 6$, *$p < 0.05$, ***$p < 0.001$ versus the corresponding value in normoxia (*dotted line*). **e** Zymograms from collected supernatants of MDA-MB-231 cells cultured for 24 or 48 h in normoxia (N) and hypoxia (H). The *bands* indicate gelatinolytic activity in the supernatants

invasive in hypoxia. This effect was significantly inhibited by chloroquine, a known inhibitor of endosomal acidification (and thereby, also TLR9-signaling) and also by a wide-spectrum MMP-inhibitor GM6001, suggesting that endosomal signaling and MMP activity may be required for the hypoxia-induced invasion in these triple-negative breast cancer cells (Supplementary Fig. S3) [27]. In order to study the role of TLR9 in this process more specifically, we performed the next experiments with the stable TLR9 siRNA MDA-MB-231 cells (described in Fig. 1) and compared their TLR9-associated gene expression and invasive behavior with that of the control siRNA MDA-

MB-231 cells in hypoxia. The hypoxia-induced TLR9 mRNA increase was ∼50 % less in the TLR9 siRNA cells, as compared with the control cells, thus further increasing the TLR9 mRNA expression gap between the control and TLR9 siRNA cells (Fig. 5a). This bigger difference at the mRNA level was now also better reflected in the TLR9 protein levels, which demonstrated a markedly increased difference between the TLR9 siRNA and control siRNA cells in hypoxia (Fig. 5b). To our surprise, the hypoxia-induced fold-increase in invasion was not blunted, but rather significantly enhanced in the TLR9 siRNA cells as compared with the control siRNA cells. Furthermore, the
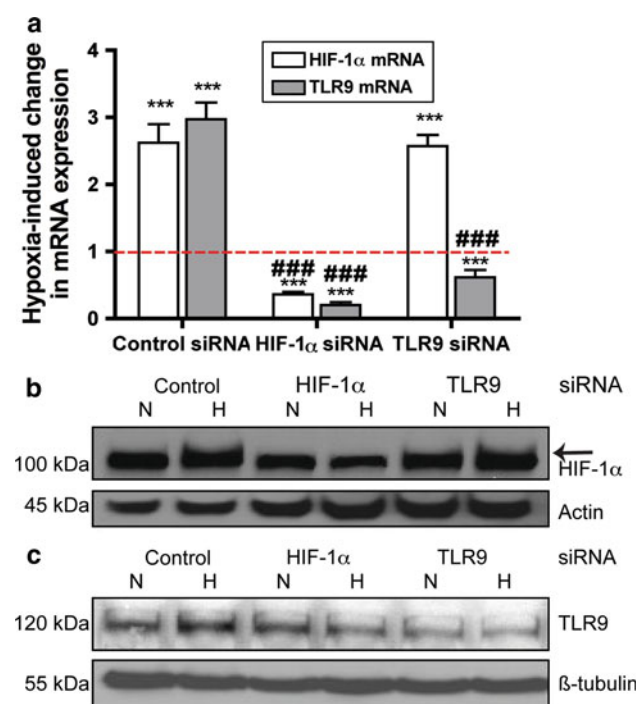
Fig. 4 **a** Hypoxia-induced changes in HIF-1α and TLR9 mRNA expression in MDA-MB-231 cells that were transiently transfected with siRNA oligonucleotides against HIF-1α, TLR9, or with control siRNA oligonucleotides. The *bars* represent hypoxia-induced changes, relative to normoxia (*dotted line*), mean ± SD, $n = 6$, ***$p < 0.001$ versus the corresponding value in normoxia, ###$p < 0.001$ versus the corresponding value in the control siRNA cells. **b** Western blots of the cells, after culture in normoxia (N) and hypoxia (H) for 24 h. Hypoxia-induced accumulation of HIF-1α protein (*arrow*) was detected in control siRNA and TLR9 siRNA cells, but not in the HIF-1α siRNA cells. The same blot was stripped and re-blotted with actin antibody to show loading. **c** Hypoxia-induced accumulation of TLR9 was detected in control siRNA cells, but not in the HIF-1α or TLR9 siRNA cells. The same blot was stripped and re-blotted with β-tubulin antibody, to show equal loading

Fig. 5 **a** Hypoxia-induced TLR9 mRNA expression in the stable, control, and TLR9 siRNA plasmid-transfected MDA-MB-231 cells. The *bars* represent hypoxia-induced changes, relative to normoxia (set to 1, indicated by the *dotted line*), mean ± SEM, $n = 6$, ***$p < 0.001$ versus normoxia, ###$p < 0.001$ versus the corresponding value in the control siRNA cells. **b** Western blot of TLR9 protein expression under hypoxic conditions in the control and TLR9 siRNA MDA-MB-231 cells. Actin band of the same, stripped blot is shown to indicate equal loading. **c** Hypoxia-induced invasion in the plasmid-based control and TRL9 siRNA cells (from **a** and **b**) and in the cells where TLR9 was down-regulated through oligonucleotide siRNAs (as in Fig. 4). The bars represent mean ± SEM, $n = 6$, *$p < 0.05$, ***$p < 0.001$ control siRNA versus TLR9 siRNA cells

invasion difference was even more pronounced in the cells where TLR9 expression was transiently blocked with oligonucleotide siRNAs (Fig. 5c). Taken together, these results suggested that decreased TLR9 expression in triple-negative breast cancer cells does not prevent hypoxia-induced invasion but rather, enhances it. In order to study these effects in ER+ cells, we also established stable T47-D control and TLR9 siRNA cells. Hypoxia also induced a slight induction of TLR9 expression in the parental T47-D and control siRNA T47-D cells, but not in the TLR9 siR-NA T47-D cells (Fig. 6a, b). Interestingly, although HIF-1α mRNA expression was not up-regulated by hypoxia in T47-D cells, HIF-1α siRNA molecules still blocked hypoxia-induced TLR9 mRNA expression, suggesting that this hypoxia-induced effect is HIF-1α-mediated also in these ER+ breast cancer cells (Supplementary Fig. S4). Hypoxia also induced parental T47-D cell invasion (Fig. 6c). The hypoxia-induced invasive response of these
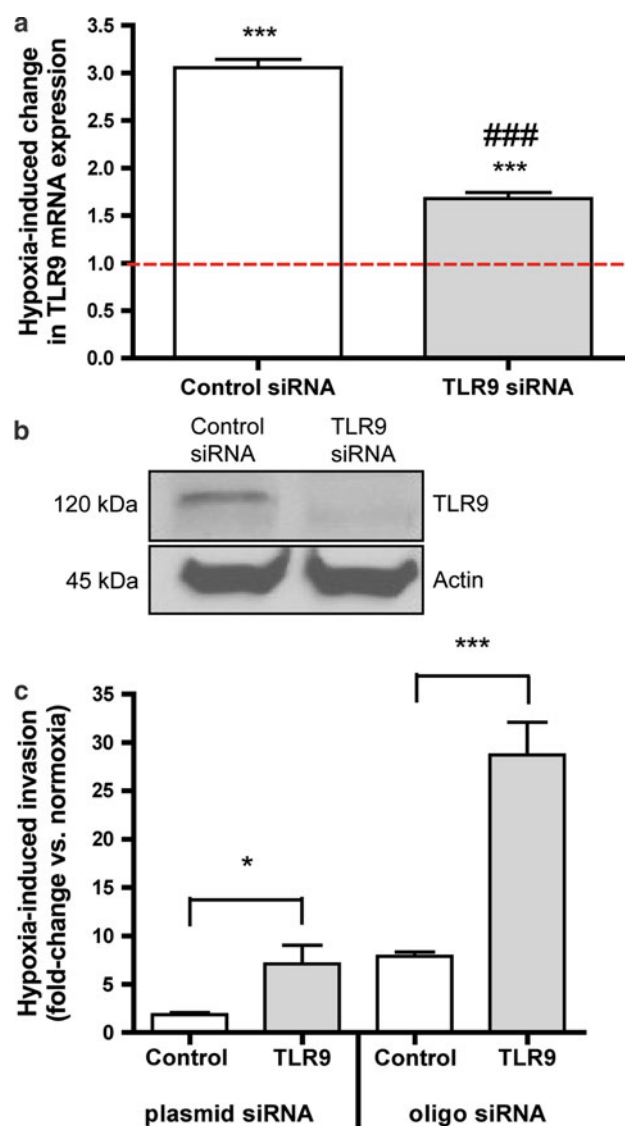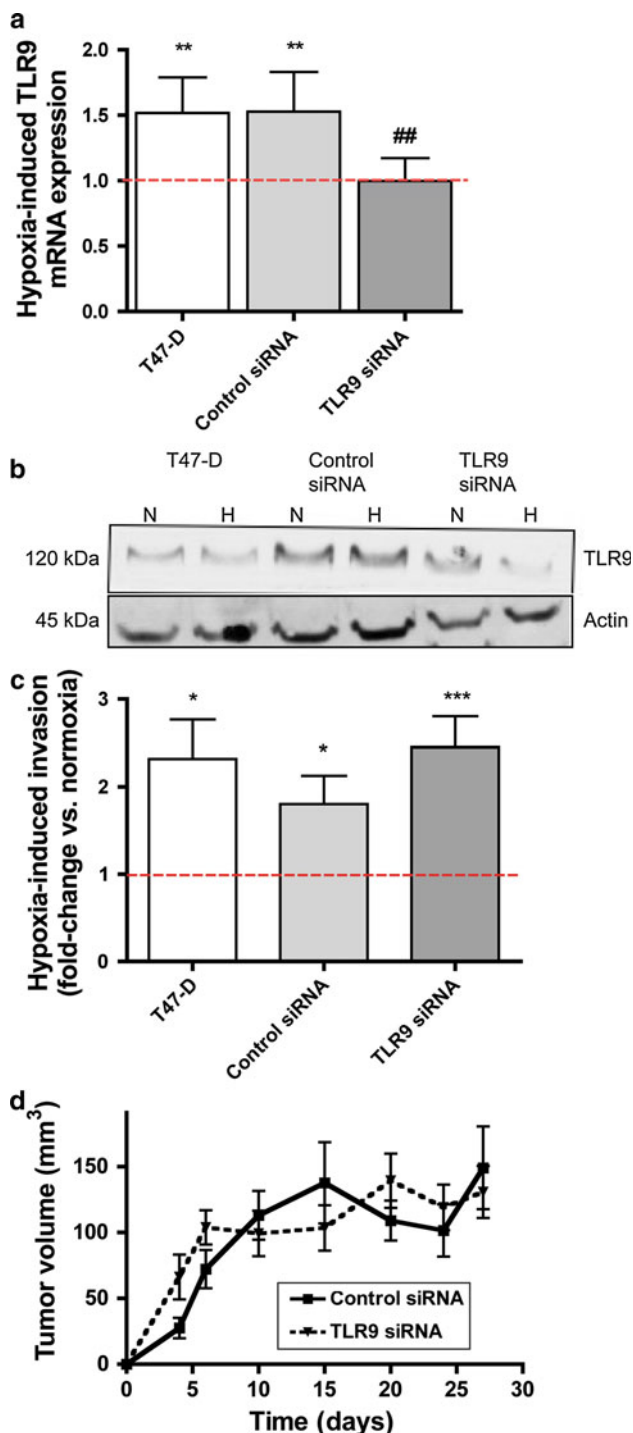
ER+, TLR9 siRNA cells was, however, similar to that detected in the control siRNA cells and unlike in the triple-negative TLR9 siRNA cells, not enhanced (Fig. 6c). Furthermore, the in vivo growth rates of T47-D control and TLR9 siRNA cells were similar (Fig. 6d). Taken together, these results suggest that decreased TLR9 expression in hypoxia strongly promotes the invasiveness of

Hypoxia abolishes TIMP-3 expression in low TLR9-expressing triple-negative breast cancer cells but not in low TLR9 expressing ER+ breast cancer cells

We next studied further the possible mechanisms that could explain the high invasiveness of the triple negative, low TLR9-expressing breast cancer cells in hypoxia. Similar to parental MDA-MB-231 cells, culture in hypoxia also up-regulated MMP-2, MMP-9 and MMP-13 mRNA expression in the control siRNA cells. These effects were, however, significantly diminished in the stable TLR9 siRNA cells. As in the parental MDA-MB-231 cells, TIMP-3 mRNA expression was also significantly decreased in the control siRNA cells in hypoxia (Fig. 7a). This effect was significantly augmented in the stable TLR9 siRNA cells, in which no TIMP-3 mRNA expression was detected. Hypoxia also induced up-regulation of TIMP-1 mRNA and no change in TIMP-2 mRNA in the control siRNA cells. In the TLR9 siRNA MDA-MB-231 cells, both TIMP-1 and TIMP-2 mRNAs were down-regulated by hypoxia, but the changes in the expression levels were not as dramatic as those for TIMP-3 (Fig. 7a). In agreement with the mRNA levels, TIMP-3 protein was not detected in control or TLR9 siRNA cells in hypoxia (Fig. 7b). Despite the relatively decreased MMP mRNA expression levels in the TLR9 siRNA cells, zymograms of hypoxic cell culture supernatants demonstrated, however, similar proteolytic activity for control siRNA and TLR9 siRNA MDA-MB-231 cells (Fig. 7c). In summary, these results suggest that although the hypoxia-induced stimulation of MMP expression is decreased in the triple-negative TLR9 siRNA cells, the proteolytic activity of these cells remains high in hypoxia, possibly due to lack of their endogenous inhibitors, such as TIMP-3. This is further supported by the significantly decreased TIMP-3 mRNA expression that was also detected in the TLR9 siRNA tumors ex vivo (Fig. 7d). Hypoxia effects on TIMP expression were also investigated in the ER+ TLR9 siRNA T47-D cells. In the parental and control siRNA T47-D cells, hypoxia significantly increased TIMP-3 mRNA expression

triple-negative breast cancer cells but not that of ER+ cells. Finally, we also studied TLR9 expression and invasive behavior in another human triple-negative breast cancer cell line, Hs578T cells. These cells also become highly invasive in hypoxia, but hypoxia does not increase their HIF-1α or TLR9 mRNA expression (Supplementary Fig. S5). These cells may thus represent a parental, unmodified example of the aggressive low-TLR9 subgroup.
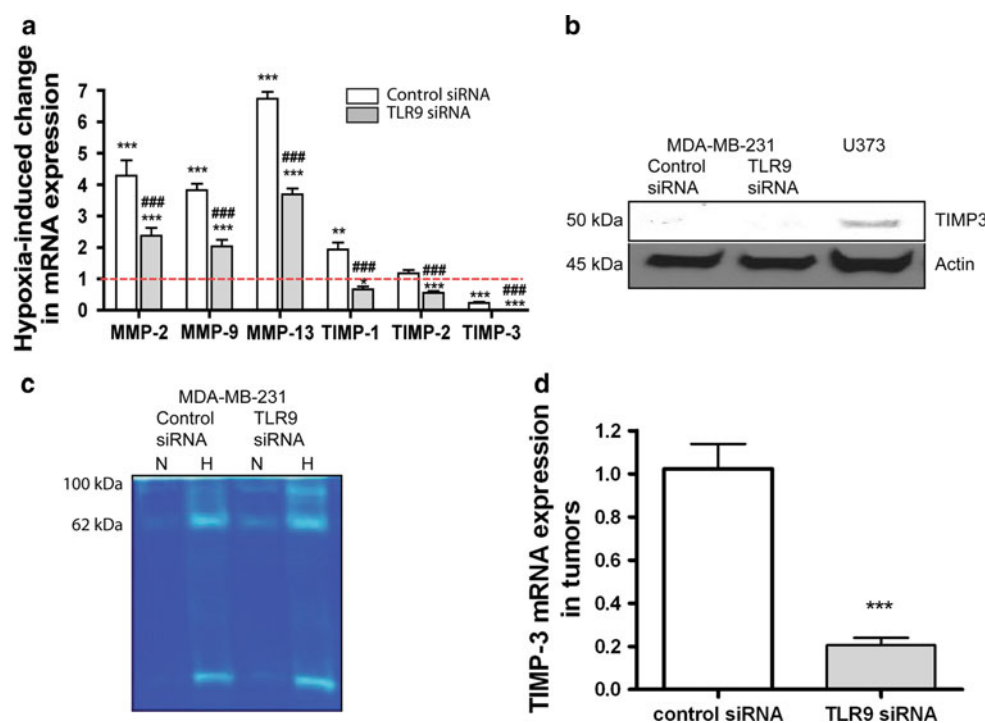
Fig. 7 a Hypoxia-induced MMP-2, MMP-9, MMP-13, and TIMP-3 mRNA expression in the plasmid-based MDA-MB-231 control or TLR9 siRNA cells. The bars represent hypoxia-induced changes, relative to normoxia (*dotted line*), mean ± SD, $n = 4$–6, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus corresponding value in normoxia, ###$p < 0.001$ versus the corresponding value in the control siRNA cells. **b** Western blot of TIMP-3 protein expression under hypoxia in the indicated cells. Actin band of the same, stripped blot is shown to indicate equal loading. Protein from U373MG glioblastoma cells was used as a positive control to demonstrate TIMP-3 protein on the blots. **c** Zymograms of supernatants, demonstrating gelatinolytic activity, from stable control or TLR9 siRNA MDA-MB-231 cells that were cultured for 24 h under normoxia (N) or hypoxia (H). **d** Relative TIMP-3 mRNA expression in control siRNA ($n = 6$) and TLR9 siRNA MDA-MB-231 tumors ($n = 12$). Mean ± SEM, ***$p < 0.001$ control siRNA versus TLR9 siRNA tumors

without affecting TIMP-1 and TIMP-2. In the T47-D TLR9 siRNA cells, the hypoxia-induced TIMP-3 mRNA up-regulation was blocked. However, even in hypoxia, the T47-D TLR9 siRNA cells retained their TIMP-3 expression (Fig. 8a, b). Zymograms of control siRNA T47-D cell supernatants exhibited a similar, hypoxia-induced increase in proteolysis as detected in parental T47-D cells. In the TLR9 siRNA T47-D cells, such change was less obvious (Fig. 8c, d).

Triple-negative TLR9 siRNA breast cancer cells exhibit increased viability in hypoxia

Finally, we tested how normoxia and hypoxia affect the cellular viability of the triple-negative breast cancer cells with high or low expression of TLR9. Similar to what was detected with the parental MDA-MB-231 cells (data not shown), the viability of the control siRNA cells was slightly increased when the cells were cultured in hypoxia. This effect was, however, significantly pronounced in the TLR9 siRNA cells (Supplementary Fig. S6).

Discussion

The majority of solid human tumors are hypoxic. The low oxygen pressure in the tumors contributes to the metastatic progression, but the molecular mechanisms involved are not thoroughly understood [28]. We demonstrate here that the innate immunity DNA receptor TLR9 is an important, oxygen status-dependent regulator of triple-negative breast cancer cell behavior. We discovered that low TLR9 expression has opposite effects on the invasive behavior of triple-negative breast cancer cells, depending on the surrounding oxygen status. In normoxic growth conditions, low TLR9 expression results in decreased invasion; while in hypoxia, low TLR9 expression is permissive for both highly increased invasion and viability. Furthermore, since the experiments were performed without the addition of exogenous ligands, our results suggest that TLR9 expression per se is sufficient to regulate invasion. Our findings further indicate that the significance of low TLR9 expression may be specific for triple-negative breast tumors, since low TLR9 expression did not augment invasiveness in ER+ breast cancer cells in vitro. While interpreting these
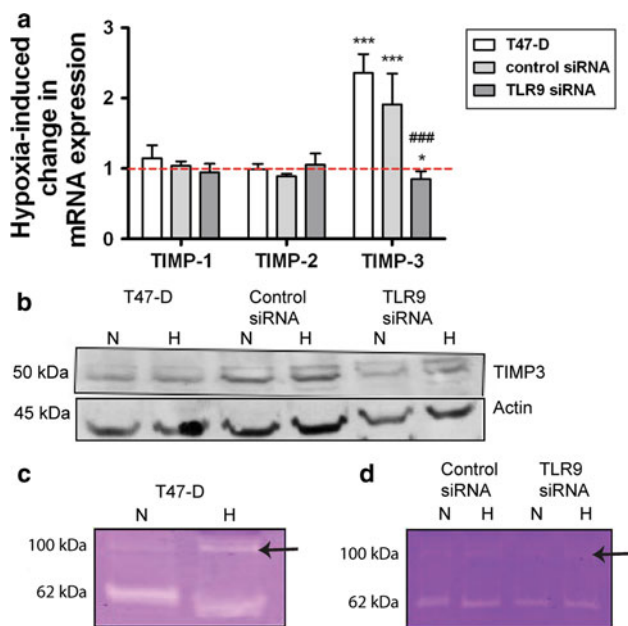
Fig. 8 **a** Hypoxia-induced changes in TIMP-1, TIMP-2, and TIMP-3 mRNA expression in parental, control siRNA, and TLR9 siRNA T47-D cells. Data is expressed as fold-change versus normoxia (*dotted line*), mean ± SD, $n = 5–6$, *$p < 0.05$, ***$p < 0.001$ versus normoxia, ###$p < 0.001$ versus corresponding control siRNA cells. **b** TIMP-3 protein expression in parental, control, and TLR9 siRNA T47-D cells in normoxia (N) and hypoxia (H). The same blot was stripped and re-blotted with anti-actin antibodies to show protein loading. Zymograms of supernatants from **c** parental T47-D and **d** stable control siRNA or TLR9 siRNA T47-D cells that were cultured for 24 h under normoxia (N) or hypoxia (H). Hypoxia-induced proteolytic bands are indicated with *arrows*

results, however, it must be taken into account that the hypoxic responses of commercially available cell lines may be very different compared to those in primary tumor cells. However, low tumor TLR9 expression predicted poor prognosis only in patients with triple-negative tumors, but not in patients with ER+ breast tumors, suggesting that low TLR9 expression might be a useful marker to distinguish those breast cancer patients with triple-negative tumors that are at high risk of relapse. The patient data further support the role of low TLR9 expression in promoting triple-negative breast cancer invasion and viability, as high tumor TLR9 expression was associated with the absence of these aggressive characteristics. These findings may not be limited to breast cancers because we also recently demonstrated a similar, decreased survival associated with low tumor TLR9 expression in renal cell carcinomas, which also have been associated with hypoxia [29].

The anti-invasive phenotype of the triple-negative TLR9 knock-out cancer cells in normoxia is likely mediated via decreased activity of the various MMPs, due to both decreased MMP expression and increased expression of their endogenous inhibitors, such as the TIMPs 1-3. In hypoxia, this TLR9-regulated balance is, however, reversed, and the dramatic decrease of endogenous MMP inhibitors, especially TIMP-3, favors the pro-invasive phenotype even when the relative, hypoxia-induced MMP expression is decreased. It is also possible that the effects on TIMP-3 and MMPs are miRNA-regulated or mediated by some another factor that gets activated in hypoxia when TLR9 expression levels are low. The molecular events that lead to increased invasion in these cells require further clarification. Interestingly, however, low TLR9 expression in hypoxia affects TIMP-3 expression differently between ER+ and triple-negative breast cancer cells; unlike the triple-negative breast cancer cells, low TLR9-expressing ER+ breast cancer cells retain their TIMP-3 expression even in hypoxia. The mechanistic explanation for this phenomenon is currently unclear, but it may be related to the ability of ERα to inhibit TLR9 signaling [30]. We showed recently that expression of ERα blocks TLR9 ligand-induced NF-κB activity and thus theoretically, ERα could also inhibit the TLR9- or possibly other TLR-regulated effects on TIMP-3 expression. This difference in TIMP-3 expression might also explain why patients with low TLR9-expressing triple-negative breast tumors do much worse than patients with ER+ low TLR9-expressing tumors. This hypothesis is supported by our ex vivo results demonstrating that TIMP-3 mRNA expression remains low in the orthotopic triple-negative TLR9 siRNA tumors. Our results from the experiments with chloroquine further suggest that in hypoxia, the pro-invasive phenotype of cells with low TLR9 may be mediated via endosomal signaling. In the absence of TLR9, hypoxia might thus activate other endosome-residing (and thus chloroquine-sensitive) TLRs, such as TLRs 3, 7, and 8. TLR9 has indeed been demonstrated to inhibit TLR7 signaling [31]. Taken together, the lack of TLR9 expression under hypoxia might thus be permissive for the pro-invasive effects of other endosomal TLRs and it may be these other factors that modulate, for example, TIMP expression. These issues call for further investigation. Finally, in addition to the intrinsic tumor invasion permitting effects of low TLR9, such phenotype might also result in increased tumor growth and poorer prognosis due to the lack of proper signals to the adaptive immunity [32]. This aspect of low TLR9 expression also needs to be resolved in further studies.

Similar to many of the hypoxia-regulated genes, hypoxia-induced up-regulation of TLR9 is dependent on HIF-1α, but not vice versa. Our findings agree with those of Sinha et al. [33], who recently demonstrated that HIF-1α also regulates TLR9 expression in glioma cells. Our findings further suggest that if the HIF-1α-mediated TLR9-response is missing, triple-negative breast cancer cells become more aggressive in hypoxia. Nuclear HIF-1α is over-expressed in primary breast cancers where it has been linked to an aggressive phenotype and poor prognosis [34]. Furthermore, deletion of HIF-1α in the mammary

epithelium resulted in decreased pulmonary metastasis in a transgenic model of cancer initiation and progression [35]. These pre-clinical findings have led to clinical testing of HIF-1α inhibitors, but conclusive results are still pending [36]. Our findings suggest that HIF-1α inhibition might be unfavorable in cancer types that have low TLR9 expression to begin with. In line with this, a naturally occurring anti-sense transcript, aHIF, whose function at the moment is unclear but which may down-regulate the expression of the mature HIF-1α protein, was recently shown to be a poor prognostic marker in a group of neo-adjuvant treated breast cancer patients [37].

Of breast cancer patients, those with triple-negative tumors typically bear the poorest prognosis, due to both aggressive behavior of the tumors and also due to the lack of targeted therapies [38]. Triple-negative breast cancers are, however, a heterogeneous group, and a more precise understanding of the various subgroups would allow rationale drug design for these patients. Our study suggests that triple-negative breast cancers with low TLR9 expression may represent a novel subgroup of highly aggressive disease. Thus, low TLR9 expression in this group might serve as a useful prognostic marker and propose that for these patients, agents that activate tumor TLR9 signaling or expression in hypoxia might improve survival. Furthermore, these results suggest that any intervention or exogenous factor that induces hypoxia or lowers TLR9 expression in residual disease or in metastatic triple-negative breast cancer cells may actually promote their spread and growth.

Materials and methods

Cell culture

Human MDA-MB-231 and Hs578T breast cancer cells were cultured in Dulbecco's modified Eagle's medium (Gibco BRL, Life Technologies, Paisley, UK) supplemented with 10 % heat-inactivated fetal bovine serum (FBS), L-glutamine, penicillin/streptomycin (PS), and non-essential amino acids (all from Gibco BRL, Life Technologies). T47-D breast cancer cells were cultured in RPMI medium, supplemented with 20 % FBS, PS, and 10 µg/mL insulin [39]. Cell culture was done in 37 °C, 5 % CO_2/95 % air ($\sim$21 % pO_2). For the hypoxia experiments, the cells were incubated at 5 % pO_2 (I-Glove, BioSpherix, Inc., Lacona, NY). Chloroquine was purchased from Sigma, and the wide-spectrum MMP-inhibitor, GM6001 from Enzo Life Sciences.

RNA isolation and quantitative RT-PCR

Total RNA was isolated from the cells using the Trizol reagent (Invitrogen) and purified with RNeasy mini kits (Qiagen). All reagents for the qRT-PCR experiments were purchased from Applied Biosystems. cDNA was synthesized from 0.2 µg of total RNA, using Multiscribe reverse transcriptase and random hexamers. Quantification of TLR9 mRNA expression was done with the following primer and probe set, which were custom made by the vendor (Applied Biosystems): forward primer (GGCCCTCCACGCATGAG), reverse primer (CTTGTCCTTTTCTGCCCTTGTAG), and probe (CCTGCAGAACTCTG). Other used primer and probe sets (CAIX, MMP-2, MMP-9, MMP-13, TIMP-1, TIMP-2, and TIMP-3) were purchased from Applied Biosystems. For all qRT-PCR assays, a standard amplification program was used (1 cycle of 50 °C for 2 min, 1 cycle of 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and 60 °C for 1 min). After normalization with RPLPO expression levels for each cDNA, a relative quantification of target cDNA was performed using $2^{-\Delta c_t}$ values.

Western blot analysis

The cells were incubated for indicated times in serum-free culture medium, the medium was discarded and the cells were harvested in lysis buffer (Cell Signaling) and clarified by centrifugation. After boiling the supernatants in reducing SDS sample buffer, equal amounts of protein ($\sim$100 µg) were loaded per lane and the samples were electrophoresed into 10 or 4–20 % gradient polyacrylamide SDS gels (BioRad) and transferred to a nitrocellulose membrane. In order to detect TLR9, the blots were incubated overnight at 4 °C with the anti-TLR9 antibodies (IMG-431, Imgenex), and diluted 1:500 in Tris-buffered saline and 0.1 % (v/v) Tween-20 (TBST). TIMP-3 was detected with a polyclonal rabbit (Millipore, AB8106, 1:500). HIF-1α protein was detected with Novus Biologicals, NB100-134, with the dilution of 1:500. Equal loading was confirmed with polyclonal rabbit anti-actin (Sigma; A-2066, 1:1000) or with anti-mouse β-tubulin antibody (D66, Sigma, 1:1000). Secondary detection was performed with HRP-linked secondary antibodies (GE Healthcare). The protein bands were visualized by chemiluminescence using ECL kit (Pierce, Rockford, IL).

Zymograms

The cells were incubated 24 h in serum-free media in normoxia or hypoxia. The supernatants were collected and concentrated with centrifugal filter device (Millipore, cut-off size 3 kDa, cat # UFC5-003-24). Equal amounts of protein ($\sim$20 µg) were loaded per lane of zymogram gels (10 % gelatin, BioRad). The gels were then run, re-naturated, and developed, using BioRad zymogram buffers, according to the manufacturer's recommendations.

RNA interference

Down-regulation of TLR9 with siRNA in the MDA-MB-231 cells was done with two approaches. In the plasmid-based approach, a previously described TLR9 siRNA sequence or a control, non-targeting siRNA sequence was cloned into the pSuper-EGFP vector (Oligoengine, Seattle, WA) [14]. The plasmids were stably transfected into MDA-MB-231 cells, using standard techniques [9]. The cells were selected in the presence of G418 (0.8 mg/mL) and three (T47-D) or four (MDA-MB-231) cycles of green fluorescence-based cell sorting was done, to obtain a pool of cells with high percentage of the cells showing GFP emission. For a short-term down-regulation of TLR9, oligonucleotides (human TLR9 siRNA Smart pool or a control, human non-targeting siRNA pool, Dharmacon) were used. In brief, 8 μL of the 20 μM siRNA stock were added to 500 μL of Optimem (Gibco) on 6-well plates, followed by addition of 5 μL of Lipofectamine RNAimax (Invitrogen). The plates containing the mixtures were rocked on a platform for 20 min at RT, followed by addition of 0.8 mL of MDA-MB-231 cell suspension (45×10^4 cells per mL). After 2 h, 1.6 mL of regular growth medium was added to the cells. The medium was changed the next day and after 48 h from the transfection, the cells were used in invasion, RNA, and protein assays.

Invasion assays

Invasion assays were done as previously described [9]. In brief, the cells (20 000 MDA-MB-231 cells or 40 000 T47-D cells per insert) were allowed to invade through the extracellular matrix-like Matrigel (BD Biosciences) in 24-well plate inserts (Beckton Dickinson Labware). The number of invaded cells was counted microscopically, after the membranes were stained with the Hema-3 set (Fisher) [10].

Cell viability assays

The cells were plated on 96-well plates (20,000 cells per 100 μL per well) in normal growth medium. The viability of the cells was measured with the CellTiter 96 Aqueus One Solution Cell Proliferation assay (Promega), according to the manufacturer's recommendations. In another set of experiments, the cells were plated on 24-well plates and after the indicated time, the cells were trypsinized, and the viable cells were counted after Trypan blue staining with the TC10™ automated cell counter (Biorad).

Animal studies

The MDA-MB-231 cells (10^6 cells in 100 μL in PBS, $n = 60$ mice) or T47-D cells [10^7 cells in 100 μL 50 % Matrigel (BD Biosciences) in PBS, $n = 12$ mice], stably transfected with the control or TLR9 siRNA plasmids, were inoculated into the mammary fat pads of four-week old, athymic nude mice (Athymic nude/nu Foxn1 mice, Harlan). For T47-D tumor-bearing mice, s.c. ER pellets (17β-estradiol, 0.72 mg, 90-day release, Innovative Research of America, OH) were applied to facilitate the in vivo tumor growth. Starting on day 2 after tumor cell inoculation, the tumor diameters were measured in two perpendicular directions and tumor volumes were calculated using the formula $V = (\pi/6)(d_1 \times d_2)^{3/2}$, where d_1 and d_2 are the perpendicular tumor diameters [40]. After 3 weeks, the mice were sacrificed and the tumors were dissected, measured, and used for RNA isolation. In another experiment, orthotopic tumors that were formed by control MDA-MB-231 cells (stably expressing the empty vector pcDNA3) were similarly inoculated and allowed to form tumors. These tumors were processed for immunohistochemical stainings for MMP-13 and TLR9. The TLR9 stainings were performed as previously described by us [15]. MMP-13 stainings were done otherwise similarly, but with the polyclonal anti-human MMP-13 antibody (Oncogene Research Products), at a 1:50 dilution; H&E-stainings were done using standard techniques. Throughout the experiments, the animals were maintained under controlled pathogen-free environmental conditions. Animal welfare was monitored daily for clinical signs. The experiment procedures were reviewed and approved by the UAB Institutional Animal Care and Use Committee (IACUC).

Clinical specimens and immunohistochemistry

The clinical samples used here are from a cohort of breast cancer patients with local or locally advanced disease ($n = 196$) that were treated at the Department of Oncology, University Hospital of Oulu or at the University Hospital of Kuopio in 2000–2008. Ninety nine of the patients had triple-negative breast cancer, defined as a total lack of ER, PR, and HER2 expression. Control cases ($n = 97$) were ER- and PR-positive and HER2-negative. Membranous HER2 expression was studied by immunohistochemistry and if a specimen exhibited a HER2-positive result (1+ to 3+ on a scale of 0 to 3+) in IHC, Her2 gene amplification status was determined by means of chromogenic in situ hybridization (CISH). Breast cancers with six or more gene copies of Her2 in cells were considered HER2 positive [41]. Immunohistochemical staining for TLR9 and HIF-1α (Novus Biologicals, NB100-134, 1:100) and scoring of the staining intensities were done as previously described [15]. TLR9-staining intensity scores (0–16) were divided into low (<8) and high (≥8), according to the previously used criteria [15]. For the HIF-1α stainings, the intensity scores (0–20) were divided into

high and low, according to the median value 14.1. Clinical information was obtained from patient records. The pathologist reviewing the samples was blinded to clinical data. The baseline patient characteristics are given in Supplementary Table S1. All patients got standard treatment and care, consisting of surgery, radiation, and adjuvant chemotherapy. Patients with ER+ tumors were also treated with standard, adjuvant hormonal therapies. The study was approved by the local ethical committees and by the Finnish Authority of Medicolegal Affairs.

Statistical analysis

The results are given as mean ± SD or mean ± SEM, as stated. Unpaired t test was used to calculate statistically significant differences in the in vitro and in vivo experiments. For the immunohistochemical staining data of the clinical specimens, SPSS 17.0.2 for Windows was applied for statistical analysis. Survival was analyzed with the Kaplan–Meier curve with log-rank test. The probability values <0.05 were considered significant.

References

1. Akira S, Hemmi H (2003) Recognition of pathogen-associated molecular patterns by TLR family. Immunol Lett 85(2):85–95
2. Wagner H (2004) The immunobiology of the TLR9 subfamily. Trends Immunol 25(7):381–386
3. Shi Z, Cai Z, Sanchez A, Zhang T, Wen S, Wang J, Yang J, Fu S, Zhang D (2011) A novel Toll-like receptor that recognizes vesicular stomatitis virus. J Biol Chem 286(6):4517–4524
4. Lande R, Gregorio J, Facchinetti V, Chatterjee B, Wang YH, Homey B, Cao W, Wang YH, Su B, Nestle FO, Zal T, Mellman I, Schroder JM, Liu YJ, Gilliet M (2007) Plasmacytoid dendritic cells sense self-DNA coupled with antimicrobial peptide. Nature 449(7162):564–569
5. West XZ, Malinin NL, Merkulova AA, Tischenko M, Kerr BA, Borden EC, Podrez EA, Salomon RG, Byzova TV (2010) Oxidative stress induces angiogenesis by activating TLR2 with novel endogenous ligands. Nature 467(7318):972–976
6. Leifer CA, Kennedy MN, Mazzoni A, Lee C, Kruhlak MJ, Segal DM (2004) TLR9 is localized in the endoplasmic reticulum prior to stimulation. J Immunol 173(2):1179–1183
7. Nishiya T, DeFranco AL (2004) Ligand-regulated chimeric receptor approach reveals distinctive subcellular localization and signaling properties of the Toll-like receptors. J Biol Chem 279(18):19008–19017
8. Schmausser B, Andrulis M, Endrich S, Lee SK, Josenhans C, Muller-Hermelink HK, Eck M (2004) Expression and subcellular distribution of toll-like receptors TLR4, TLR5 and TLR9 on the gastric epithelium in Helicobacter pylori infection. Clin Exp Immunol 136(3):521–526
9. Ilvesaro JM, Merrell MA, Swain TM, Davidson J, Zayzafoon M, Harris KW, Selander KS (2007) Toll like receptor-9 agonists stimulate prostate cancer invasion in vitro. Prostate 67(7):774–781
10. Merrell MA, Ilvesaro JM, Lehtonen N, Sorsa T, Gehrs B, Rosenthal E, Chen D, Shackley B, Harris KW, Selander KS (2006) Toll-like receptor 9 agonists promote cellular invasion by increasing matrix metalloproteinase activity. Mol Cancer Res 4(7):437–447
11. Droemann D, Albrecht D, Gerdes J, Ulmer AJ, Branscheid D, Vollmer E, Dalhoff K, Zabel P, Goldmann T (2005) Human lung cancer cells express functionally active Toll-like receptor 9. Respir Res 6:1
12. Schmausser B, Andrulis M, Endrich S, Muller-Hermelink HK, Eck M (2005) Toll-like receptors TLR4, TLR5 and TLR9 on gastric carcinoma cells: an implication for interaction with Helicobacter pylori. Int J Med Microbiol 295(3):179–185
13. Ren T, Xu L, Jiao S, Wang Y, Cai Y, Liang Y, Zhou Y, Zhou H, Wen Z (2009) TLR9 signaling promotes tumor progression of human lung cancer cell in vivo. Pathol Oncol Res 15(4):623–630
14. Ilvesaro JM, Merrell MA, Li L, Wakchoure S, Graves D, Brooks S, Rahko E, Jukkola-Vuorinen A, Vuopala KS, Harris KW, Selander KS (2008) Toll-like receptor 9 mediates CpG oligonucleotide-induced cellular invasion. Mol Cancer Res 6(10):1534–1543
15. Jukkola-Vuorinen A, Rahko E, Vuopala KS, Desmond R, Lehenkari PP, Harris KW, Selander KS (2008) Toll-like receptor-9 expression is inversely correlated with estrogen receptor status in breast cancer. J Innate Immun 1(1):59–68
16. Väisänen MR, Väisänen T, Jukkola-Vuorinen A, Vuopala KS, Desmond R, Selander KS, Vaarala MH (2010) Expression of toll-like receptor-9 is increased in poorly differentiated prostate tumors. Prostate 70(8):817–824
17. Berger R, Fiegl H, Goebel G, Obexer P, Ausserlechner M, Doppler W, Hauser-Kronberger C, Reitsamer R, Egle D, Reimer D, Muller-Holzner E, Jones A, Widschwendter M (2010) Toll-like receptor 9 expression in breast and ovarian cancer is associated with poorly differentiated tumors. Cancer Sci 101(4):1059–1066
18. Takala H, Kauppila JH, Soini Y, Selander KS, Vuopala KS, Lehenkari PP, Saarnio J, Karttunen TJ (2011) Toll-like receptor 9 is a novel biomarker for esophageal squamous cell dysplasia and squamous cell carcinoma progression. J Innate Immun 3(6):631–638
19. Gonzalez-Reyes S, Marin L, Gonzalez L, Gonzalez LO, del Casar JM, Lamelas ML, Gonzalez-Quintana JM, Vizoso FJ (2010) Study of TLR3, TLR4 and TLR9 in breast carcinomas and their association with metastasis. BMC Cancer 10:665
20. Ruan K, Song G, Ouyang G (2009) Role of hypoxia in the hallmarks of human cancer. J Cell Biochem 107(6):1053–1062
21. Li XF, Carlin S, Urano M, Russell J, Ling CC, O'Donoghue JA (2007) Visualization of hypoxia in microscopic tumors by immunofluorescent microscopy. Cancer Res 67(16):7646–7653
22. Tan EY, Yan M, Campo L, Han C, Takano E, Turley H, Candiloro I, Pezzella F, Gatter KC, Millar EK, O'Toole SA, McNeil CM, Crea P, Segara D, Sutherland RL, Harris AL, Fox SB (2009) The key hypoxia regulated gene CAIX is upregulated in basal-like breast tumours and is associated with resistance to chemotherapy. Br J Cancer 100(2):405–411
23. Yan M, Rayoo M, Takano EA, Fox SB (2009) BRCA1 tumours correlate with a HIF-1alpha phenotype and have a poor prognosis through modulation of hydroxylase enzyme profile expression. Br J Cancer 101(7):1168–1174

24. Kuhlicke J, Frick JS, Morote-Garcia JC, Rosenberger P, Eltzschig HK (2007) Hypoxia inducible factor (HIF)-1 coordinates induction of Toll-like receptors TLR2 and TLR6 during hypoxia. PLoS One 2(12):e1364

25. Pringle KG, Kind KL, Sferruzzi-Perri AN, Thompson JG, Roberts CT (2010) Beyond oxygen: complex regulation and activity of hypoxia inducible factors in pregnancy. Hum Reprod Update 16(4):415–431

26. Munoz-Najar UM, Neurath KM, Vumbaca F, Claffey KP (2006) Hypoxia stimulates breast carcinoma cell invasion through MT1-MMP and MMP-2 activation. Oncogene 25(16):2379–2392

27. Yasuda H, Leelahavanichkul A, Tsunoda S, Dear JW, Takahashi Y, Ito S, Hu X, Zhou H, Doi K, Childs R, Klinman DM, Yuen PS, Star RA ((2008)) Chloroquine and inhibition of Toll-like receptor 9 protect from sepsis-induced acute kidney injury. Am J Physiol Renal Physiol 294(5):F1050–F1058

28. Chaudary N, Hill RP (2006) Hypoxia and metastasis in breast cancer. Breast Dis 26:55–64

29. Ronkainen H, Hirvikoski P, Kauppila S, Vuopala KS, Paavonen TK, Selander KS, Vaarala MH (2011) Absent Toll-like receptor-9 expression predicts poor prognosis in renal cell carcinoma. J Exp Clin Cancer Res 30:84

30. Sandholm J, Kauppila JH, Pressey C, Tuomela J, Jukkola-Vuorinen A, Vaarala M, Johnson MR, Harris KW, Selander KS (2012) Estrogen receptor-alpha and sex steroid hormones regulate Toll-like receptor-9 expression and invasive function in human breast cancer cells. Breast Cancer Res Treat 132(2):411–419

31. Wang J, Shao Y, Bennett TA, Shankar RA, Wightman PD, Reddy LG (2006) The functional effects of physical interactions among Toll-like receptors 7, 8, and 9. J Biol Chem 281(49):37427–37434

32. DeNardo DG, Coussens LM (2007) Inflammation and breast cancer. Balancing immune response: crosstalk between adaptive and innate immune cells during breast cancer progression. Breast Cancer Res 9(4):212

33. Sinha S, Koul N, Dixit D, Sharma V, Sen E (2011) IGF-1 induced HIF-1alpha-TLR9 cross talk regulates inflammatory responses in glioma. Cell Signal 23(11):1869–1875

34. Bos R, van der Groep P, Greijer AE, Shvarts A, Meijer S, Pinedo HM, Semenza GL, van Diest PJ, van der Wall E (2003) Levels of hypoxia-inducible factor-1alpha independently predict prognosis in patients with lymph node negative breast carcinoma. Cancer 97(6):1573–1581

35. Liao D, Corle C, Seagroves TN, Johnson RS (2007) Hypoxia-inducible factor-1alpha is a key regulator of metastasis in a transgenic model of cancer initiation and progression. Cancer Res 67(2):563–572

36. Kimbro KS, Simons JW (2006) Hypoxia-inducible factor-1 in human breast and prostate cancer. Endocr Relat Cancer 13(3):739–749

37. Cayre A, Rossignol F, Clottes E, Penault-Llorca F (2003) aHIF but not HIF-1alpha transcript is a poor prognostic marker in human breast cancer. Breast Cancer Res 5(6):R223–R230

38. Foulkes WD, Smith IE, Reis-Filho JS (2010) Triple-negative breast cancer. N Engl J Med 363(20):1938–1948

39. Neve RM, Chin K, Fridlyand J, Yeh J, Baehner FL, Fevr T, Clark L, Bayani N, Coppe JP, Tong F, Speed T, Spellman PT, DeVries S, Lapuk A, Wang NJ, Kuo WL, Stilwell JL, Pinkel D, Albertson DG, Waldman FM, McCormick F, Dickson RB, Johnson MD, Lippman M, Ethier S, Gazdar A, Gray JW (2006) A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. Cancer Cell 10(6):515–527

40. Wärri AM, Huovinen RL, Laine AM, Martikainen PM, Härkönen PL (1993) Apoptosis in toremifene-induced growth inhibition of human breast cancer cells in vivo and in vitro. J Natl Cancer Inst 85(17):1412–1418

41. Isola J, Tanner M, Forsyth A, Cooke TG, Watters AD, Bartlett JM (2004) Interlaboratory comparison of HER-2 oncogene amplification as detected by chromogenic and fluorescence in situ hybridization. Clin Cancer Res 10(14):4793–4798